BLOOD CLOTTING EFFECT IN VITRO AND WOUND HEALING ACTIVITY IN VIVO OF FRESH JUICE AND ETHANOLIC EXTRACTS OF *CROTON MACROSTACHYUS LEAVES* IN MALE WISTAR ALBINO RATS

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Declaration

I, here undersigned, declare that this MSc. thesis is my original work and has not been presented for any degree in any other university and all sources of materials used for this project have been duly acknowledged.

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Acronyms

ABS: Ankaferd Blood Stopper

ADP: Adenosine Diphosphate

DHA: Docosahexaenoic acid.

DMSO: Dimethyl sulfoxide

ELISA: Enzyme-linked immunosorbent assay

ECM: Extracellular Matrix

EPD: Eicosapentaenoic acid

HMW: High Molecular Weight

IGF-1: Insulin like Growth Factor-1

EGF: Epidermal Growth Factor

IL-1: Interleukin-1

MMP: Matrix metalloproteases

RND: Ribonucleic acid

DNA: Deoxyribonucleic acid

ATP: Adenosine triphosphate

rpm: revolution per minute

mM: mill molar

MPH: Microporous polysaccharide haemospheres

MSCs: Mesenchymal Stem Cells

NO: Nitric Oxide

TxA2: Thromboxane A_2

PDGF: Platelet-Derived Growth Factor

PFDs: Platelets Function Disorders

PGI_2: Prostacyclin

PUFAs: Polyunsaturated fatty acids

TF: Tissue Factor

TGF-β: Transforming Growth Factor beta

TNF-α: Tumor necrosis factor-alpha

vWF: von Willebrand factor

WHO: World Health Organization

Abstract

Background: The plant remedies (both single plant and multi-herbal preparations) are used to promote wound healing since ancient times even if the mechanisms of action, toxicity and efficacy of very few of them have been evaluated scientifically. Preparations from plant sources have been traditionally considered effective to stop bleeding and promote wound healing. Juice extract from leaves of *Croton macrostachyus* has been traditionally used as a local haemostatic medicine to hasten clotting, however, this effect had not been tested in controlled experiments.

Aim: There was no scientific evidence justifying the use of *Croton macrostachyus*, therefore the present study was aimed at evaluation of platelets aggregating and wound healing activity of the plant.

Materials and methods: In the present study the Leaves of *Croton macrostachyus* were studied for procoagulant and wound healing activity by using ethanolic and fresh juice extract. The procoagulant activity was studied by using the effect of extracts on platelets aggregation. Wound healing activity was studied in excision model in rats, wound contraction, period of epithelization, hydroxyproline estimation and histopathological examinations were studied.

Results: Ethanolic extract induced significant ($P<0.05$) platelets aggregation where as the fresh juice extract did not induce significant platelets aggregation. Platelets aggregation induced by ethanolic extract significantly increased as the time of contact increased ($P<0.05$). Treatment of wound with ointment containing 10% (w/w) of the ethanolic extract and 10% (w/v) of the fresh juice extract exhibited significant ($P < 0.05$) wound healing activity.

Conclusion: The ethanolic extract of *Croton macrostachyus* leaves has procoagulant activity in that it promotes platelets aggregation in vitro that verifies its effect on haemostasis. Moreover, ethanolic extract of the plant leaves promoted wound healing in vivo that could be explained partly by its procoagulant activity and the aqueous juice extract had promoting effect on wound healing that could be explained by the multiple chemical constituents which are of water soluble.

Key words: *Croton macrostachyus*, Platelets aggregation, Procoagulant activity, wound healing, Excision, wound, Hydroxyproline.

1. Introduction

1.1. Background

Medicinal plants are of great importance to the health of individuals and communities in general. The medicinal value of plants lies in some chemical substances that produce a definite physiological action on the human body (Omale and Emmanuel, 2010). The use of herbal medicine for the treatment of diseases and infections is as old as mankind. The World Health Organization supports the use of traditional medicine provided they are proven to be efficacious and safe. In developing countries, a huge number of people lives in extreme poverty and some are suffering and dying for want of safe water and medicine, they have also no alternative for primary health care (WHO, 1985).

Therefore, the need to use medicinal plants as alternatives to modern medicines in the provision of primary health care has been emphasized. More so herbal medicines have received much attention as sources of lead compounds since they are considered as time tested and relatively safe for both human use and environment friendly (Fazly *et al.*, 2005). They are also cheap, easily available and affordable. Many medicinal plants are claimed to be useful for wound healing in the traditional system of medicine. These plant remedies (both single plant and multi-herbal preparations) are used since ancient times even if the mechanisms of action, toxicity and efficacy of very few of them have been evaluated scientifically (Ximena and Luis, 2003). Many researchers reported that plants such as *Aloe Vera, Peperomia galioides, Anredera diffusa, Calendula officinalis, Pentas lanceolata* and *Jatropha curcas* have prohealing actions (Nayak and Krishna, 2007).

A wound is defined as a break in the epithelial integrity of the skin. However, the disruption could be deeper, extending to the dermis, subcutaneous fat, fascia, muscle or even the bone. This disruption is due to any causes such as ulcers, burns, neoplasm or traumas and as a result normal functions are no longer adequately performed (Stuart and Patricia, 2004). Wound may be produced by chemical, physical, thermal, microbial or immunological insult to the tissue. Wound cause discomfort and are more prone to infection and other troublesome complications (Omale and Isaac, 2010). It is therefore vital to restore its integrity as soon as possible. Normal wound

healing involves a complex and dynamic but superbly orchestrated series of events leading to the repair of injured tissues. A completely healed wound, usually seen after simple injury, is defined as one that has returned to its normal anatomical structure, function and appearance within a reasonable period of time. In contrast to these some wounds fail to heal in a timely and orderly manner, resulting in chronic, non-healing wounds (Stuart and Patricia, 2004).

Healing of wounds occurs as a sequential cascade of overlapping processes that requires the coordinated completion of a variety of cellular activities including phagocytosis, chemotaxis, mitogenesis, collagen synthesis and the synthesis of other matrix components. These activities do not occur in a haphazard manner but rather in a carefully regulated and systematic cascade that correlates with the appearance of different cell types in the wound during various stages of the healing process. These processes, which are triggered by tissue injury, involve the four overlapping but well-defined phases of haemostasis, inflammation, proliferation and remodeling (Clark, 1996).

Wound care and maintenance involve a number of measures including dressing and administration of painkillers, use of anti-inflammatory agents, topical and systemic antimicrobial agents and healing promoting drugs. A large number of materials have been reported to affect healing differentially. A treatment could influence the healing of wounds by intervening in one or many phases of wound healing. However, the intensive research in wound healing has not yielded, until today, a safe, economic and efficacious pro-healing agent that could obviate the long hospitalization of patients following surgeries, wounds etc (Nayak and Krishna, 2007). The use of efficacious haemostatic agents lead to less tissue trauma, decrease bleeding and improve out comes (Michael and Dean, 2006). Effective hemostasis is an important requirement of dermatologic surgery because the results of failed hemostasis can include persistent bleeding, delayed wound healing, skin necrosis, wound dehiscence, and infection (Palm and Altman, 2008). Juice extract from leaves of *Croton macrostachyus* has been traditionally used as a local haemostatic medicine to hasten clotting, however, the literature survey revealed that no systematic study had been carried out on its wound healing activity.

1.2. Botanical and Ecological information of *Croton Macrostachyus*

Croton macrostachyus is commonly known as rush foil or broad-leaved Croton in English and Bisana in Amharic (Breitenbach, 1963). It belongs to the Euphorbiaceae, a very large family with 300 genera and 8,000 to 10,000 species, and is the most numerous in the tropics (Heywood, 1993). The name of the genus *Croton* comes from a Greek word *Kroton*, which means ticks, because of the seeds' resemblance to ticks. The genus contains over 1,200 species, which are distributed throughout the world (Mairura, 2007). Eight of these species (*C. dichogamus*, *C. zambesicus*, *C. menyhartii*, *C. somalense*, *C.schimperianus*, *C. sylvaticus*, *C. lobatus*, and *C. macrostachyus*) are found in Ethiopia, but the most common species is *Croton macrostachyus* (Gilbert, 1995). The name of the species is from the Greek macro – (large) and – stachyus (relating to spike), hence, macrostachyus means "with a large spike" (Amare, 2010).

Croton macrostachyus is native to Eritrea, Ethiopia, Kenya, Tanzania, Uganda and Nigeria. It is a medium sized deciduous tree of East Africa particularly wide spread between 200-2500 m in mountainous forests and savannah of the tropical regions and ever green bush land areas that receive between 700-2000 mm rainfalls annually (Kapingu *et al.*, 2000).

1.3. The role *Croton macrostachyus* in traditional medicine

In Kenya the Luhya people lick the ash of burnt leaves as a cough remedy. A leaf decoction is also taken to treat cough and stomach problems. A root decoction is taken to treat indigestion. The Kikuyu people of Kenya take a root decoction to treat malaria; leaf juice is put on wounds to improve blood clotting, and also to treat sores, warts and ringworm (Matu & van Staden, 2003). In Kenya and Tanzania a root infusion is taken to treat intestinal parasites. A decoctions of the stem and root bark are used for bathing babies with skin rash. A bark infusion is taken to treat chest problems and rheumatism (Mairura, 2007).

Roots and leaves Decoction is taken for treatment of Pneumonia, backache, cancer, dry cough, obesity, malaria, skin diseases, typhoid, sorcery and used as purgative in Nandi district of Kenya (Jeruto *et al.*, 2010).

In Ethiopia *Croton macrostachyus* has many uses. A leaf extract is applied against itchy scalp. A decoction of the leafy twigs mixed with *Justicia schimperiana* is taken to treat jaundice and smallpox. The preparation is taken with pepper, butter and milk. An infusion of the leafy branches and roots is used as a mouthwash to treat toothache. The leaves or young shoots are eaten to treat fever and edema and mashed leaves are applied to haemorrhoids. A preparation of the seed is instilled into the ear to treat ear problems. The seeds are poisonous and are used to make fish poison, while crushed seed and leaves in water are taken to treat tapeworm infection; the seed is eaten to induce abortion and a fruit, bark or root decoction or raw fruit is taken to treat venereal diseases. Bark maceration is drunk as an abortifacient and uterotonic, to expel a retained placenta. These treatments are all considered dangerous. The root or stem bark is chewed to treat toothache, but also rabies. Ripe crushed fruits mixed with butter or honey and ground leaves are applied to skin diseases (Mairura, 2007). Moreover, the juice from fresh leaves of *Croton macrostachyus* is applied on bleeding wounds to hasten clotting (Amare, 2010; Teshale *et al.*, 2004).

1.4. Phytochemical information of *Croton macrostachyus*

Much research has not been done concerning the chemical composition of Croton macrostachyus (Mairura, 2007). But phytochemical study on the genus *Croton* has lead to the isolation and characterization of different classes of secondary metabolites. Terpenes, flavonoids and alkaloids have been isolated from the different *croton* species. Terpenoids are the predominant secondary metabolite constituents in the genus, chiefly diterpenoids, which may belong to the clerodane, neoclerodane, kaurane, labdane, phorbol and trachylobane skeletal types. Triterpenoids, either pentacyclic or steroidal, have frequently been reported for *croton* species. Apparently, clerodane is the widest spread class of diterpenoids in croton, which has been found in species from America (e.g. *C. cajucara*), Africa (e.g. *C. macrostachyus*) and Asia (e.g. *C. tiglium*) (Amare, 2010; Zelalem, 2007).

Several species of the genus are aromatic, indicating the presence of volatile oil constituents. As most Euphorbiaceae, croton species may contain latex, which is red-coloured in some species, a characteristic usually with medicinal properties (Amare, 2010).

According to the study by Amare (2010), the concentration of eleven metals, essential (Ca, Mg, Mn, Fe, Cu, Zn, Co, Cr, Ni) and non-essential (Pb, Cd) in the *Croton macrostachyus* leaves were determined by Flame Atomic Absorption Spectrometry .These work described that the samples from different area had variable composition of each analyte metals with wide concentration range. However, the concentrations of metals in *Croton macrostachyus* were not investigated by multiple works to support this evidence.

1.5. Wound Healing

Wound healing is an intricate process in which the skin (or another organ-tissue) repairs itself after injury. In normal skin, the epidermis (outermost layer) and dermis (inner or deeper layer) exists in steady-state equilibrium, forming a protective barrier against the external environment. Once the protective barrier is broken, the normal (physiologic) process of wound healing is immediately set in motion. The classic model of wound healing is divided into three or four sequential, yet overlapping, phases: (1) hemostasis (2) inflammatory, (3) proliferative and (4) remodeling. Upon injury to the skin, a set of complex biochemical events takes place in a closely orchestrated cascade to repair the damage. Within minutes post-injury, platelets (thrombocytes) aggregate at the injury site to form a fibrin clot. This clot acts to control active bleeding (hemostasis) (Stuart and Patricia 2004; JoAn *et al.,* 2003) (figure 1.1).

In the inflammatory phase, bacteria and debris are phagocytosed and removed, and factors are released that cause the migration and division of cells involved in the proliferative phase. The proliferative phase is characterized by angiogenesis, collagen deposition, granulation tissue formation, epithelization, and wound contraction. In angiogenesis, new blood vessels are formed by vascular endothelial cells. In fibroplasia and granulation tissue formation, fibroblasts grow and form a new, provisional extracellular matrix (ECM) by excreting collagen and fibronectin (JoAn *et al.,* 2003).

Concurrently, re-epithelization of the epidermis occurs, in which epithelial cells proliferate and providing cover for the new tissue. The wound also undergoes physical contraction throughout the entire wound healing process, which is believed to be mediated by contractile fibroblasts (myofibroblasts) that appear in the wound (Gosain and DiPietro, 2004; Campos *et al*., 2008). In the maturation and remodeling phase, collagen is remodeled and realigned along tension lines and cells that are no longer needed are removed by apoptosis. However, this process is not only complex but fragile, and susceptible to interruption or failure leading to the formation of non-healing chronic wounds.

The progression of acute wound healing from hemostasis to the final phases of remodeling is dependent on a complex interplay of varied acute wound-healing events. Cytokines play a central role in wound healing and serve as a central signal for various cell types and healing events (JoAn *et al.,* 2003).

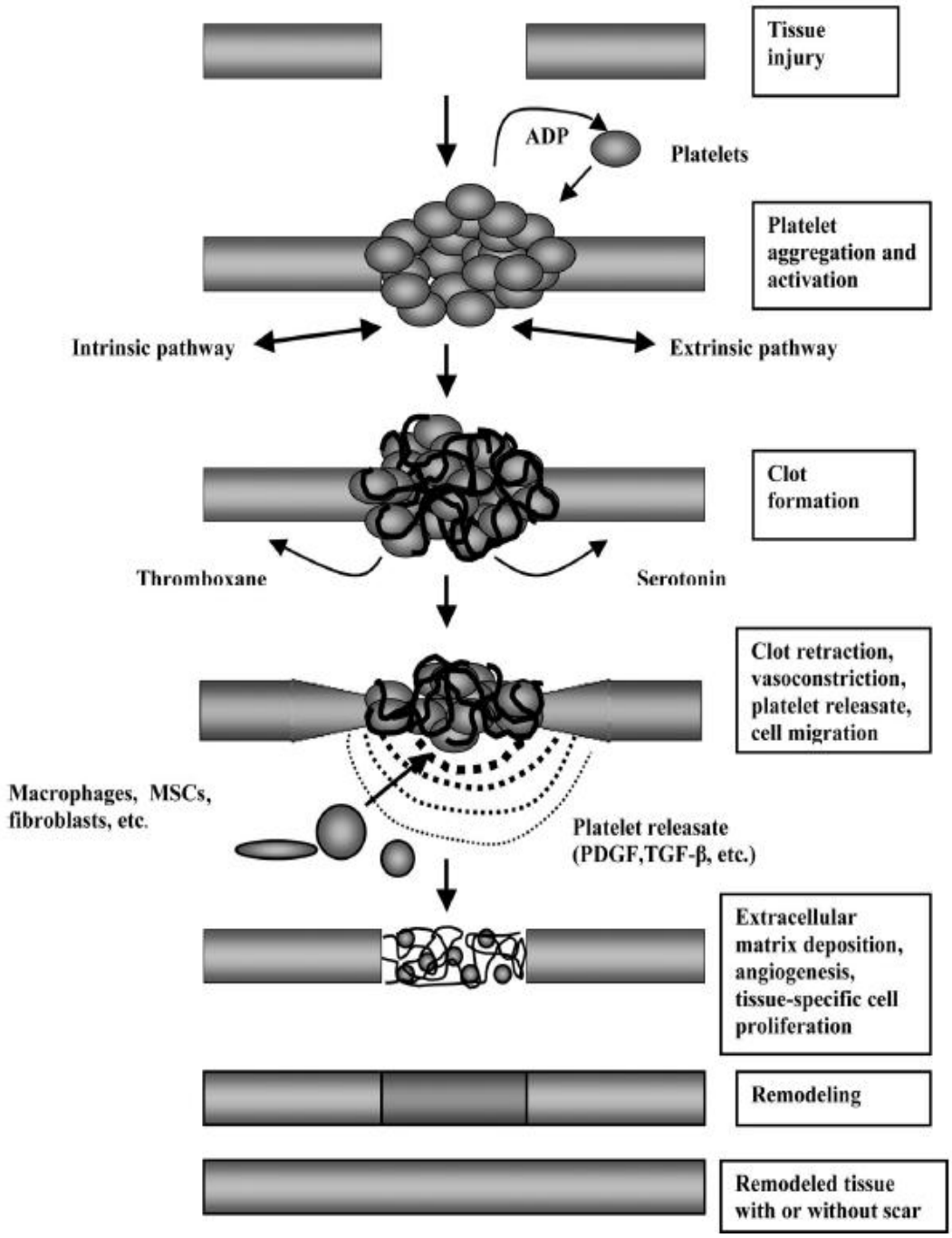

Figure1.1. Schematic diagram of wound healing. MSCs, mesenchymal stem cells; PDGF, platelet-derived growth factor; TGF-β, transforming growth factor β (Barry et al., 2006).

1.5. 1. Haemostasis

Haemostasis is an important biological response to wounding or injury that prevents excessive blood loss. All significant traumas create a vascular injury and thereby initiate the molecular and cellular responses that establish haemostasis. The wound healing process cannot proceed until haemostasis is accomplished (JoAn *et al.*, 2003). Primary contributors to hemostasis include vasoconstriction, platelet aggregation, and fibrin deposition resulting from the coagulation cascades. The end product of the haemostatic process is clot formation. Clots are primarily composed of fibrin mesh and aggregated platelets along with embedded blood cells (Lawrence, 1998). The importance of clot formation is profound. This process prevents further fluid and electrolyte loss from the wound site and limits contamination from the outside environment. Fibrin is also the mesh material in the provisional wound matrix onto which fibroblasts and other cells migrate as the healing process proceeds. In the event of hemorrhage, hemostasis is naturally carried out by vessel contraction, platelet aggregation, activation of coagulation factors and blood flow (Seyednejad *et al.*, 2008).

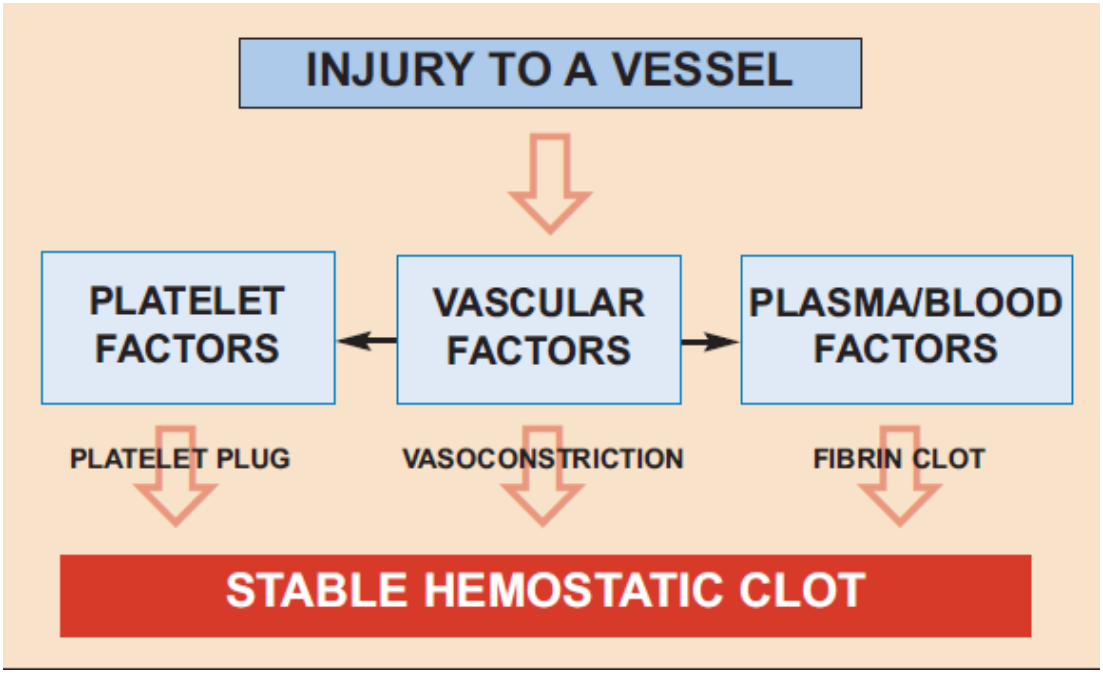

Figure 1.2. The synergy of factors that contribute to haemostasis (Srinath, 2008)

Vasoconstriction is initiated by the release of vasoactive amines, which occurs when the dermis is penetrated. Epinephrine is released into the peripheral circulation, whereas stimulation of the sympathetic nervous system results in local norepinephrine release. Injured cells secrete prostaglandins, such as thromboxane, that contribute further to vasoconstriction (JoAn *et al.*, 2003).

Platelets are the primary cells involved in mediating the thrombotic response. They are central mediators of haemostasis at sites of vascular injury, but they also mediate pathologic thrombosis. The coagulation cascade culminates in the formation of the master enzyme thrombin, which once formed can activate platelets, as well as participate in its own production and destruction. In addition to thrombin formed downstream of the coagulation cascade, platelets are efficiently activated by collagen exposed at the site of injury. Moreover, platelets can self activate via released ADP or thromboxane. Mediators like prostacyclin (PGI_2) or Nitric Oxide (NO) from the intact endothelium can down- regulate platelet activation and keep them quiescent. But once the platelets encounter a net activating stimulus as a function of the concentration of all of these simultaneous signals they proceed via a sequence of signaling steps (one amongst which is the elevation of cytosolic calcium concentration) to become fully activated platelets. These platelets make up the body of clot and are held in place by a meshwork of polymerized fibrinogen formed downstream of thrombin in the coagulation cascade (Manash, 2011)

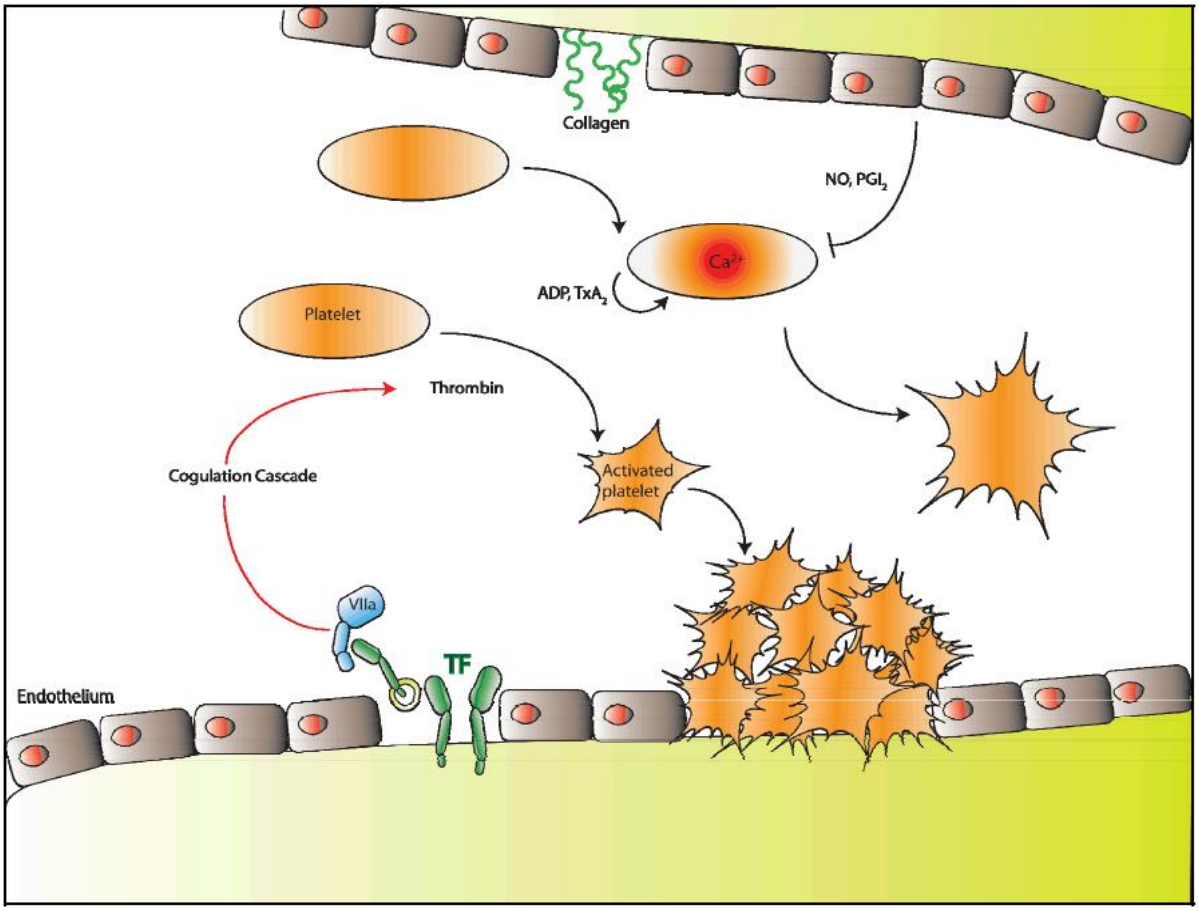

Figure 1.3. Coagulation and platelet activation pathways during thrombosis. TF, tissue factor; ADP, Adenosine diphosphate; TxA2, Thromboxane $A_{2,}$ (Manash, 2011).

The platelets trapped in the clot are essential for haemostasis as well as for a normal inflammatory response. The alpha granules of the platelets contain growth factors, including platelet-derived growth factor (PDGF), insulin-like growth factor-1 (IGF-1), epidermal growth factor (EGF), and transforming growth factor-β (TGF-β). These proteins initiate the wound healing cascade by attracting and activating fibroblasts, endothelial cells and macrophages. The platelets also contain dense bodies that store vasoactive amines such as serotonin that increase microvascular permeability. This leads to the exudation of fluid into the extravascular space and results in tissue edema, although this feature is more prominent during the inflammatory phase (Stuart and Patricia, 2004).

When a body tissue is injured and begins to bleed, it initiates a sequence of clotting factor activities, the coagulation cascade-leading to the formation of a blood clot. This cascade is comprised of three pathways: extrinsic, intrinsic, and common. Coagulation begins almost instantly after an injury to the blood vessel has damaged the endothelium. Platelets immediately form a plug at the site of injury (primary hemostasis). Secondary hemostasis occurs simultaneously. Proteins in the blood plasma (clotting factors) respond with a complex cascade to form fibrin strands which strengthen the platelet plug (Michael and Dean, 2006).

The coagulation cascade is a series of dependent reactions involving several plasma proteins, calcium ions, and blood platelets that lead to the conversion of fibrinogen to fibrin (Sabel and Stummer, 2004). Coagulation factors are produced by the liver and circulate in an inactive form until the coagulation cascade is initiated. Vessel or endothelial cell injury stimulates a cascade of events mediated predominantly by serine proteases, in which inactive zymogens are proteolytically cleaved to act on the next precursor downstream in the pathway. A crucial step in the coagulation cascade is activation of prothrombin by a prothrombinase activator complex (consisting of factors Xa and Va bound in the presence of calcium ions (Ca^{2+}) on a phospholipid membrane) to produce thrombin. Accordingly, thrombin cleaves fibrinogen into fibrin and activates Factor XIII, which cross-links fibrin to form a stable clot. Factor Xa alone can activate prothrombin into thrombin, but only at a very slow rate. In the presence of Factor Va, Ca^{2+} and phospholipid, prothrombin activation by Factor Xa is enhanced by several orders of magnitude. (Krebs et al., 2006). In the final steps, thrombin converts the soluble plasma protein fibrinogen to the insoluble protein fibrin, while simultaneously converting factor XIII to factor XIIIa. This factor conversion stabilizes the fibrin and results in cross-linking of the fibrin monomers, producing a stable clot (Sabel and Stummer, 2004) (figure 1.4).

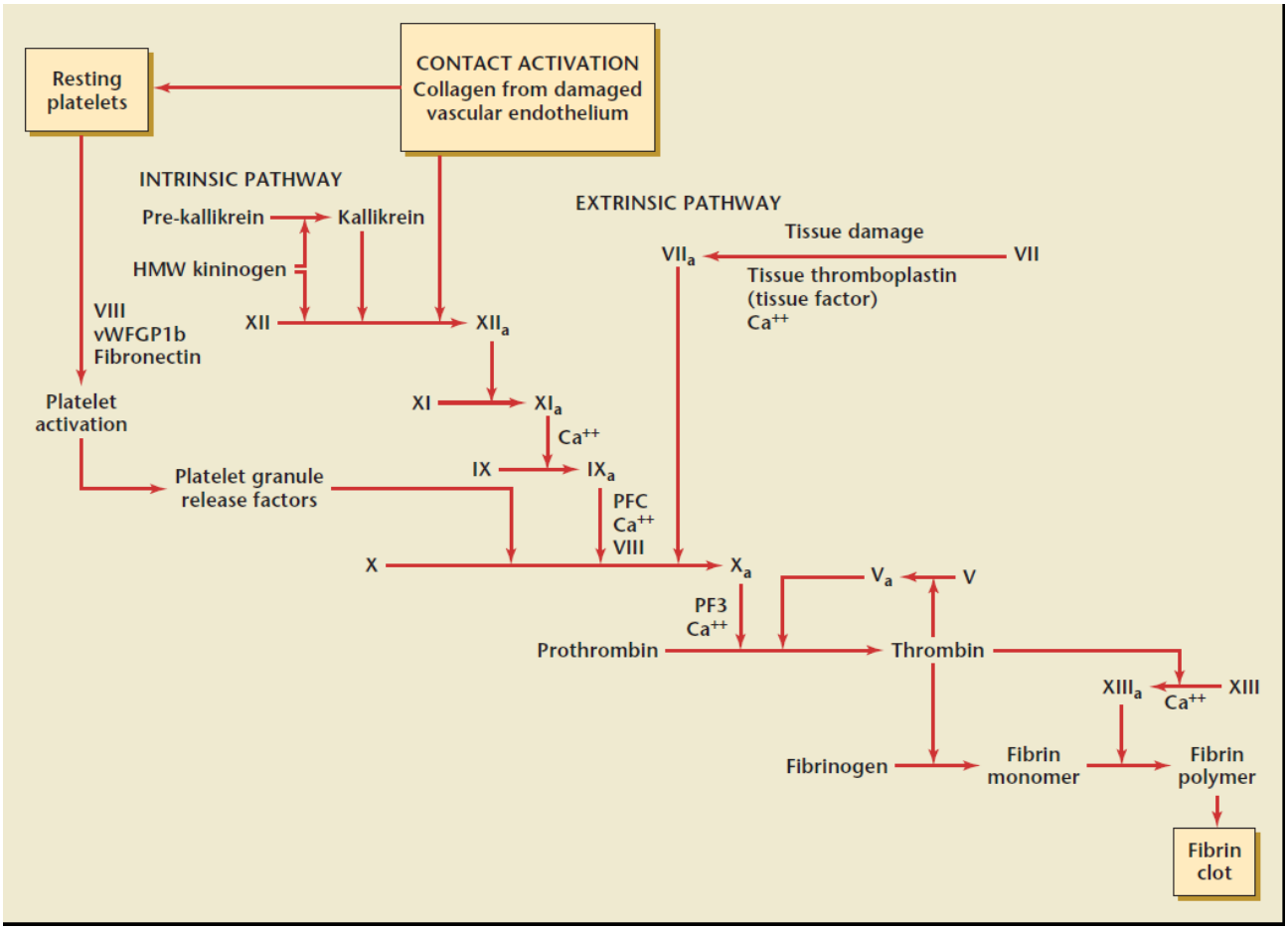

Figure 1.4. Coagulation cascade. HMW, high molecular weight (Michael and Dean, 2006).

1.5.2. Hemorrhage (haemostatic failure)

Hemorrhage occurs after disruption of the vascular wall and it is the greatest threat to survival in the first 24 hours following traumatic injury (Bijan and Harold, 2004).It is the main reason that causes death in 48 hours after trauma, which accounts for 80% in all trauma accident(Bochu et al., 2011). In surgical patients, there is potential for hemorrhage anytime an incision is made into the skin, subcutaneous tissue, muscle, and organ parenchyma. Excessive surgical bleeding causes hypovolaemia, haemodynamic instability, anemia and reduced oxygen delivery to tissues with a subsequent increase in postoperative morbidity and mortality (Mahdy and Webster, 2004). Blood loss, while minor in every day cuts and bruises, is one of the main causes of mortality. Hemorrhage threatens the life safety of patients and the wounded in trauma care and surgical intervention (Bochu *et al.*, 2011). Haemostatic failure, is also a serious problem encountered in dentistry, can cause

excessive postoperative bleeding, delayed wound healing, and increased risk of infection (Yavuz *et al.*, 2010). It accounts for nearly 50% of all deaths on the battlefield and 39% of civilian trauma deaths, most of which occur before patients reach the hospital. The use of haemostatic agents for trauma injury has mostly been undertaken in the military setting (Bijan and Harold 2004).

Platelet and other coagulation factors play an important role in the haemostasis mechanism. Inherited deficiencies of plasma proteins involved in blood coagulation and inherited platelet function disorders (PFDs) generally lead to lifelong bleeding disorders, whose severity is directly proportional to the degree of factor deficiency (Firdos *et al.,* 2008). Manifestation of Inherited haemostasis defect is seen with the patient who has suffered apparently excessive or frequent unprovoked bleeding, most commonly epistaxis or menorrhagia, or at parturition. They also suffer unexpected or apparently excessive bleeding after an intervention, such as dental extraction, post-surgical bleeding and due to traumatic injuries (Greaves and Watson, 2007).

The expanding use of anticoagulants and platelet inhibitor drugs has resulted in an increased proportion of the population being at risk of abnormal bleeding. Knowledge of the levels of risk associated with particular drugs and combinations, and the advantages and hazards of interruption of drug use for planned interventional procedures, are essential in order to reduce the incidence of iatrogenic bleeding (Greaves and Watson, 2007). In addition, systemic anticoagulation and the ubiquitous use of antiplatelet agents in the setting of vascular and cardiac surgeries increase the risk for intraoperative and postoperative bleeding (Whitlock *et al.,* 2005).

Liver disease may leads to difficulty in producing proteins involved in blood clotting. This may be due to the liver failure itself, or to a decreased absorption of vitamin K, which is needed for the production of many clotting factors. This leads to reduced clotting levels and abnormal clotting function. Bleeding in kidney disease is primarily caused by a defect of the function of platelets and blood vessel walls due to a buildup of waste products in the blood which are normally excreted in urine. As a consequence of uremia, platelets become dysfunctional and their interaction with other substances is impaired (Michael and Dean, 2006).

1.5.3. Haemostatic agents

During excessive bleeding (hemorrhage), it is not possible to wait for the natural haemostatic process to occur, and therefore, additive methods to obtain a stable coagulum have to be used. In general, these methods can be classified into three basic categories: thermal, mechanical or chemical means (Kemal *et al.*, 2010). Mechanical means include manual pressure, ligature and the application of a tourniquet. However, these methods can be labor intensive and add time to the operative procedure. Sealing of bleeding vessels can also be achieved by thermal methods such as electro cauterization or laser cauterization, but these create areas of char and necrotic tissue, increasing the likelihood of infection and damaging wound edges. This may lead to impaired healing. Conventional methods are also less effective in controlling bleeding from complex injuries and where access to the area of bleeding is difficult. Topical haemostatic agents may be particularly useful in such situations (Seyednejad *et al.*, 2008).

Several topical haemostatic agents are currently available in a range of configurations. They exert their effect in a variety of ways. Some improve primary haemostasis, whereas others stimulate fibrin formation or inhibit fibrinolysis. Some are a preparation of a procoagulant substance in combination with a vehicle such as collagen matrix. Others use a matrix to provide a template for the endogenous coagulation cascade to achieve haemostasis (Seyednejad *et al.*, 2008).

Topically applied haemostatic agents have been used in human and veterinary surgery for many years. Materials that can achieve intraoperative haemostasis include bone wax, gelatin sponges, oxidized regenerated cellulose, collagen sponges, microfibrillar collagen, topical thrombin, fibrin sealants, and, microporous polysaccharide powder. These topical agents have a wide range of clinical applications and are highly effective in obtaining rapid, sustained hemostasis (Wagner *et al.*, 1996).

Extracts from several plants, such as *Sanguisorba officinalis, Sophora japonica, Nelumbo nucifera* have been shown to have haemostatic effects to varying degrees (Liao *et al.*, 2008). However, Ankaferd Blood Stopper (ABS) as a traditional Turkish herbal medicine extract seems

to have more practical implications in the field of medicine for controlling bleeding. ABS is a standardized mixture of five plants *Thymus vulgaris, Glycyrrhiza glabra, Vitis vinifera, Alpinia officinarum,* and *Urtica dioica*. Through its effects on the endothelium, blood cells, angiogenesis, cellular proliferation, vascular dynamics, and cell mediators, ABS is now becoming an official alternative haemostatic medicine for intractable bleedings that are resistant to conventional anti-hemorrhagic measures in Turkey. Furthermore, ABS seems to have a considerable therapeutic benefit, because of its anti-infective, antineoplastic, and wound healing properties, to restore and maintain tissue homeostasis in a variety of diseases (Yavuz *et al.,* 2010).

The ideal topical haemostatic agent has high haemostatic action, minimal tissue reactivity, nonantigenicity, in vivo biodegradability, ease of sterilization, low cost, and can be tailored to specific needs. No single haemostatic agent is ideal for all situations, but existing products should be carefully chosen for a given operative environment (Palm and Altman, 2008).

Fortunately, characterization of the biochemistry of the endogenous coagulation system has made it possible to develop targeted strategies to enhance haemostatic control in a variety of surgical settings (Grant, 2007). In particular, during the past 2 decades, biologic haemostatic agents that mimic and enhance stages of the coagulation cascade have emerged (Palm and Altman, 2008) (figure 1.5).

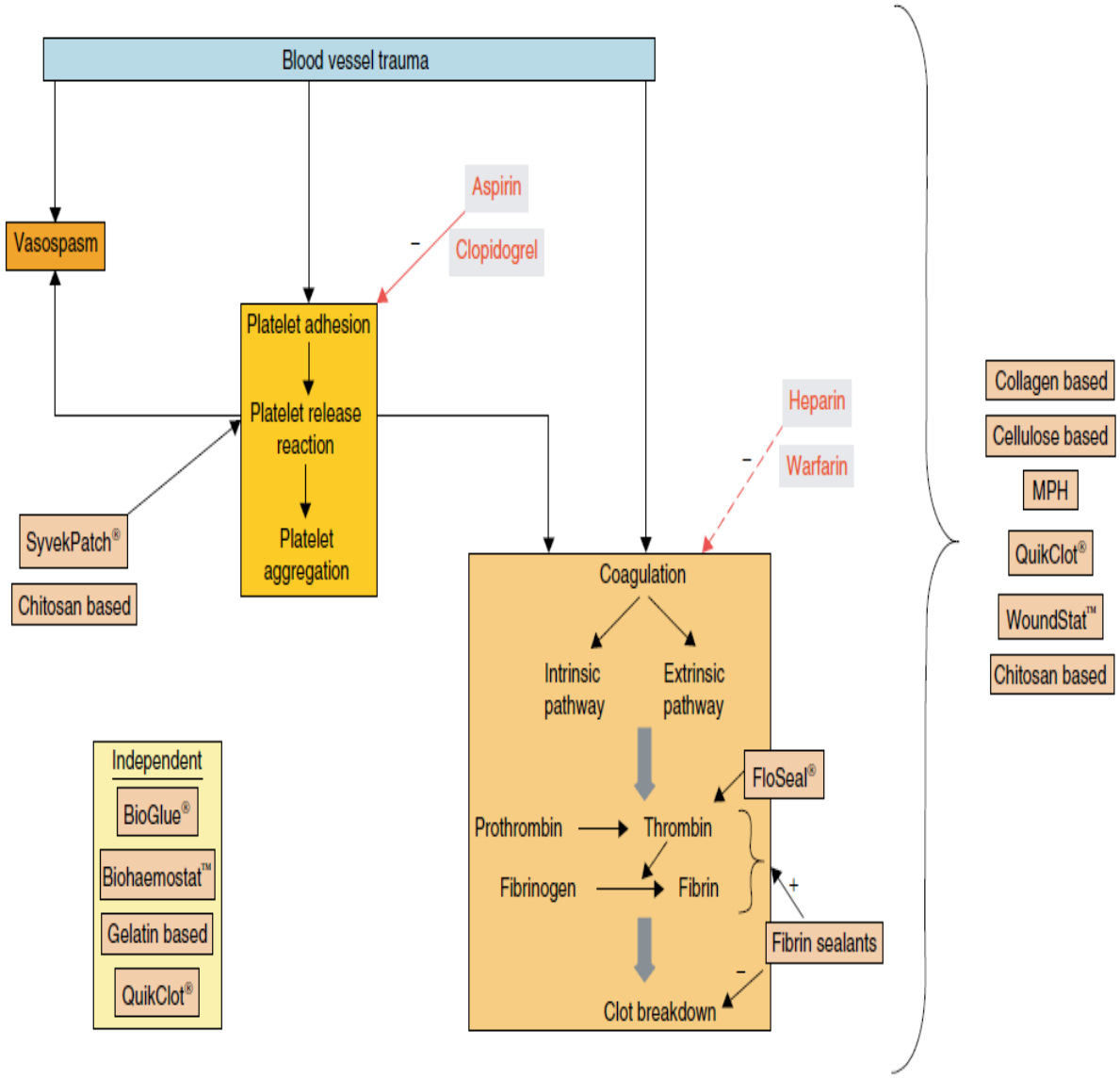

Figure.1.5. Mechanisms of action of haemostatic agents. The relationship between different topical haemostatic agents and various components of the clotting cascade is shown, along with some commonly used anticlotting agents. Those on the right are thought to depend to some extent on the entire clotting cascade. MPH, microporous polysaccharide haemospheres (*Seyednejad et al., 2008).*

1.6. Other agents important for wound healing

1.6.1. Antimicrobial agents

Once skin is injured, micro-organisms that are normally sequestered at the skin surface obtain access to the underlying tissues. The states of infection and replication status of the microorganisms determine whether the wound is classified as having contamination, colonization, local infection/critical colonization, and/or spreading invasive infection. Contamination is the presence of non-replicating organisms on a wound, while colonization is defined as the presence of replicating microorganisms on the wound without tissue damage. Local infection/ critical colonization are an intermediate stage, with microorganism replication and the beginning of local tissue responses. Invasive infection is defined as the presence of replicating organisms within a wound with subsequent host injury (Edwards and Harding, 2004).

Inflammation is a normal part of the wound-healing process, and is important to the removal of contaminating micro-organisms. In the absence of effective decontamination, however, inflammation may be prolonged, since microbial clearance is incomplete. Both bacteria and endotoxins can lead to the prolonged elevation of pro-inflammatory cytokines such as interleukin-1 (IL-1) and TNF-α and elongate the inflammatory phase. If this continues, the wound may enter a chronic state and fail to heal. This prolonged inflammation also leads to an increased level of matrix metalloproteases (MMPs), a family of proteases that can degrade the Extracellular matrix (ECM). In tandem with the increased protease content, a decreased level of the naturally occurring protease inhibitors occurs. This shift in protease balance can cause growth factors that appears in chronic wounds to be rapidly degraded (Edwards and Harding, 2004; Menke *et al*., 2007). Similar to other infective processes, the bacteria in infected wounds occur in the form of biofilms, which are complex communities of aggregated bacteria embedded in a self secreted extracellular polysaccharide matrix (Edwards and Harding, 2004). Mature biofilms develop protected microenvironments and are more resistant to conventional antibiotic treatment. *Staphylococcus aureus* (*S. aureus*), *Pseudomonas aeruginosa* (*P. aeruginosa*), and β-hemolytic *streptococci* are common bacteria in infected and clinically non-infected wounds (Edwards and Harding, 2004; Davis *et al*., 2008).

Therefore, topical application of anti-microbial agents and other chemo-therapeutic agents is essential in order to restore the normal healing process and prevent infection. Topical antimicrobial agents include both antiseptics and antibiotics. Antiseptics are agents that destroy or inhibit the growth and development of microorganisms in or on living tissue. Unlike antibiotics, which act selectively on a specific target, antiseptics have multiple targets and a broader spectrum of activity that includes bacteria, fungi, viruses and protozoa.

Many essential oils possess antimicrobial properties, and tea tree oil in particular (derived from the Australian native plant *Melaleuca alternifolia*) has been recognized for its effective antibacterial role (Livingstone *et al.,* 1990).

Honey is an ancient remedy gaining renewed popularity as an alternative treatment for infections caused by antibiotic-resistant bacteria. Both honey and sugar (in a paste form) are considered useful as topical antimicrobial agents, primarily as a consequence of their high osmolarity and ability to minimize water availability to bacteria (Carson et al ., 1998).Although the dilution of honey in the presence of wound fluid is likely to reduce the efficacy of its osmotic effect, the slow and sustained production of hydrogen peroxide by some types of honey (e.g., manuka honey) is capable of maintaining an antimicrobial effect at a concentration approximately 1000-fold lower (and less toxic) than that commonly used in antiseptics (i.e., 3%)(Malon, 1999). Also, components of manuka honey, such as flavonoids and aromatic acids, demonstrate antimicrobial properties (Cooper and Malan, 1999). Honey may also serve as a wound deodorizing agent; this effect is attributed to the glucose that is metabolized by bacteria as opposed to proteinaceous necrotic tissue, resulting in the production of lactic acid and not the malodorous compounds generated by protein degradation. Honey's usefulness in infected wounds has been attributed to its high glucose content and low pH, both of which stimulate macrophages (Moch *et al.,* 1999).

1.6.2 Nutrition

For more than 100 years, nutrition has been recognized as a very important factor that affects wound healing. Most obvious is that malnutrition or specific nutrient deficiencies can have a profound impact on wound healing after trauma and surgery. Patients with chronic or non-healing wounds and experiencing nutrition deficiency often require special nutrients. Energy, carbohydrate, protein, fat, vitamin, and mineral metabolism all can affect the healing process (Arnold and Barbul, 2006).

Carbohydrates

Together with fats, carbohydrates are the primary source of energy in the wound-healing process. Glucose is the major source of fuel used to create the cellular ATP that provides energy for angiogenesis and deposition of the new tissues (Shepherd, 2003). The use of glucose as a source for ATP synthesis is essential in preventing the depletion of other amino acid and protein substrates (Arnold and Barbul, 2006).

Proteins

Protein is one of the most important nutrient factors affecting wound healing. A deficiency of protein can impair capillary formation, fibroblast proliferation, proteoglycan synthesis, collagen synthesis, and wound remodeling. A deficiency of protein also affects the immune system, with resultant decreased leukocyte phagocytosis and increased susceptibility to infection (Gogia, 1995). Collagen is the major protein component of connective tissue and is composed primarily of glycine, proline, and hydroxyproline. Collagen synthesis requires hydroxylation of lysine and proline, and co-factors such as ferrous iron and vitamin C. Impaired wound healing results from deficiencies in any of these co-factors (Campos *et al*., 2008).

Arginine is a semi-essential amino acid that is required during periods of maximal growth, severe stress, and injury. Arginine has many effects in the body, including modulation of immune function, wound healing, hormone secretion, vascular tone, and endothelial function. Arginine is also a precursor to proline, and, as such, sufficient arginine levels are needed to support collagen deposition, angiogenesis, and wound contraction (Shepherd, 2003; Campos *et*

al., 2008). Arginine improves immune function, and stimulates wound healing in healthy and ill individuals (Tong and Barbul, 2004). Under psychological stress situations, the metabolic demand of arginine increases, and its supplementation has been shown to be an effective adjuvant therapy in wound healing (Campos *et al*., 2008).

Glutamine is the most abundant amino acid in plasma and is a major source of metabolic energy for rapidly proliferating cells such as fibroblasts, lymphocytes, epithelial cells, and macrophages (Arnold and Barbul, 2006; Campos *et al*., 2008). The serum concentration of glutamine is reduced after major surgery, trauma, and sepsis, and supplementation of this amino acid improves nitrogen balance and diminishes immuno suppression (Campos *et al*., 2008). Glutamine has a crucial role in stimulating the inflammatory immune response occurring early in wound healing (Arnold and Barbul, 2006). Oral glutamine supplementation has been shown to improve wound breaking strength and to increase levels of mature collagen (da Costa *et al*., 2003).

Fatty Acids

Lipids are used as nutritional support for surgical or critically ill patients to meet energy demands and provide essential building blocks for wound healing and tissue repair. Polyunsaturated fatty acids (PUFAs), which cannot be synthesized *de novo* by mammals, consist mainly of two families, n-6 (ω -6, found in soybean oil) and n-3 (ω -3, found in fish oil). The biochemical metabolism of ω -6 fatty acids like linoleic acid produces eicosanoids in the body. Eicosanoids are biologically active and contribute to the formation of thrombi and atheromas and shifts the physiological state to one that is prothrombotic and proaggregatory, with increases in blood viscosity, vasospasm, and vasoconstriction and decreases in bleeding time (Simopoulos, 2008). Fish oil has been widely touted for the health benefits of ω -3 fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The effects of ω -3 fatty acids on wound healing are not conclusive. They have been reported to affect pro-inflammatory cytokine production, cell metabolism, gene expression, and angiogenesis in wound sites (McDaniel *et al*., 2008; Shingel *et al.*, 2008). The true benefit of ω -3 fatty acids may be in their

ability to improve the systemic immune function of the host, thus reducing infectious complications and improving survival (Arnold and Barbul, 2006).

Vitamins and Trace elements

Vitamins C (L-ascorbic acid), A (retinol), and E (tocopherol) show potent anti-oxidant and anti-inflammatory effects. Vitamin C has many roles in wound healing, and a deficiency in this vitamin has multiple effects on tissue repair. Vitamin C deficiencies result in impaired healing, and have been linked to decreased collagen synthesis and fibroblast proliferation, decreased angiogenesis, and increased capillary fragility. Also, vitamin C deficiency leads to an impaired immune response and increased susceptibility to wound infection (Arnold and Barbul, 2006; Campos *et al*., 2008). Similarly, vitamin A deficiency leads to impaired wound healing. The biological properties of vitamin A include anti-oxidant activity, increased fibroblast proliferation, modulation of cellular differentiation and proliferation, increased collagen and hyaluronate synthesis, and decreased MMP- mediated extracellular matrix degradation (Burgess, 2008).

Vitamin E, an anti-oxidant, maintains and stabilizes cellular membrane integrity by providing protection against destruction by oxidation. Vitamin E also has anti-inflammatory properties and has been suggested to have a role in decreasing excess scar formation in chronic wounds. Animal experiments have indicated that vitamin E supplementation is beneficial to wound healing (Arnold and Barbul, 2006; Burgess, 2008), and topical vitamin E has been widely promoted as an anti-scarring agent. However, clinical studies have not yet proved a role for topical vitamin E treatment in improving healing outcomes (Khoosal and Goldman, 2006).

Several micronutrients have been shown to be important for optimal repair. Magnesium functions as a co-factor for many enzymes involved in protein and collagen synthesis, while copper is a required co-factor for cytochrome oxidase, for cytosolic anti-oxidant superoxide dismutase, and for the optimal cross-linking of collagen. Zinc is a co-factor for both RNA and DNA polymerase, and a zinc deficiency causes a significant impairment in wound healing. Iron is required for the hydroxylation of proline and lysine, and, as a result, severe iron deficiency can result in impaired collagen production (Shepherd, 2003; Arnold and Barbul, 2006; Campos *et al.*, 2008).

1.6.3. Oxygen

Oxygen is important for cell metabolism, especially energy production in the form of ATP, and is critical for nearly all wound healing processes. It prevents wounds from infection, induces angiogenesis, increases keratinocyte differentiation, migration, and re-epithelization, enhances fibroblast proliferation and collagen synthesis, and promotes wound contraction (Bishop, 2008; Rodriguez *et al*., 2008). In addition, the level of superoxide production (a key factor for oxidative killing pathogens) by polymorphonuclear leukocytes is critically dependent on oxygen levels.

Due to vascular disruption and high oxygen consumption by metabolically active cells, the microenvironment of the early wound is depleted of oxygen and is quite hypoxic. Several systemic conditions, including advancing age and diabetes, can create impaired vascular flow, thus setting the stage for poor tissue oxygenation. In the context of healing, this overlay of poor perfusion creates a hypoxic wound. Chronic wounds are notably hypoxic; tissue oxygen tensions have been measured transcutaneously in chronic wounds from 5 to 20 mm Hg, in contrast to control tissue values of 30 to 50 mm Hg (Tandara and Mustoe, 2004).

1.7. Hypothesis

The purpose of this study was to test the following hypothesis:

- Fresh juice and ethanolic extracts of Croton macrostachyus leaves may have procoagulant activity in vitro and wound healing effect in vivo.

2. Objectives

2.1. General Objective

To evaluate procoagulant activity and wound healing effects of *Croton macrostachyus* fresh juice and ethanolic leaves extracts in male Wistar albino rats.

2.2. Specific objectives

- To determine the effect of *Croton macrostachyus* leaves extracts on platelets aggregation in vitro.
- To assess the effect of *Croton macrostachyus* leaves extracts on wound healing in vivo.

3. Methods and Materials

3.1 Collections and preparation of plant materials

Enough amounts of the leaves of *Croton macrostachyus* were collected from Gamo Gofa zone, South nations' nationalities peoples region, 545 kilometers south of Addis Ababa. Before collection, the plant materials were identified and authenticated by a taxonomist in Addis Ababa University, Department of botanical sciences.

Figure 3.1 Leaves of Croton macrostachyus(photograph).

3.2. Study design and set up

Ethanolic extraction experimentation was done in Ethiopian Nutrition and Health Research Institution (ENHRI), Traditional and Modern Drug Research Directorate. All the other experiments were done in Core laboratory and Biochemistry Department in Addis Ababa University, Tikur Anbessa Teaching Specialized Hospital.

3.3. Preparation of fresh juice

The collected plant fresh leaves were washed with water and copped in to small pieces and crushed by mortar and pestle and socked with double distil water. The fresh juice was filtered off by using whatman filter paper no. 1 and transferred into a clean closed container and were kept in refrigerator at 8 degree centigrade until further use.

3.4. Preparation of ethanolic leaves extract

The leaves were made to dry under shade and powdered by electrical mill. Powdered leaves were extracted by macerating with70% ethanol for 72 hours and then the mixture was filtered using Whatman filter paper no. 1. The filtrate was made ethanol free by evaporating it using rotary vaporizers under reduced pressure at a temperature of 40^{O}c. It was kept in refrigerator at 8 degree centigrade and fresh stock solutions were prepared for the experiment whenever required.

3.5. Experimental Animals

Male adult Wister rats weighing 250-350 g were used for the study. The animals were purchased from Ethiopian Nutrition and Health Research Institution (ENHRI). The animals were kept under standard conditions (at a room temperature and with 12 hr light/ 12 hr dark cycle) and provided with free access to standard pellet laboratory diet and water ad libitum during the whole period of the study.

3.6. Methods and materials for in vitro study

3.6.1 Platelets aggregation test

Principle

Platelet aggregation testing measures the ability of various agonists on platelets to induce *in vitro* activation and platelet-to-platelet activation. When an agonist is added the platelets aggregate and absorb less light and so that the transmission increases where as the absorbance decrease. This can be measured spectrophotometrically.

Procedures

The platelet aggregating activity was measured by spectrophotometric method as described by Sophia et al., (2005). Blood was collected in anticoagulant solution (2.4% sodium citrate, 1.5% citric acid and 1.8% dextrose) by cardiac puncture. The ratio of the blood to anticoagulant solution was approximately 6:1 and the platelet rich plasma (PRP) was separated by centrifugation at 2000 rpm for 7 minutes. The platelet rich plasma was then suspended in a buffer composed of 109 mM NaCl, 4.3 mM K_2HPO_4, 16 mM Na_2HPO_4, 8.3 mM NaH_2PO_4 and 5.5 mM dextrose, pH 7.5. To 250µL of platelet suspension, 5µL of the sample extracts (0.125,

0.25, 0.5, 1.0 and 5.0 mg/mL ethanolic extract dissolved in DMSO and aqueous juice extract) were added and 5µL of DMSO was added for control group. The optical density at 600 nm was measured at three minutes interval for duration of 12 minutes after addition of samples using ELISA plate wells. For each category five replicates were done.

3.7. Methods and materials for in vivo study

3.7.1 Ointment preparation

Ointments were formulated using liquid paraffin as a solvent. 10% w/v and 10% v/v of dried ethanolic extract and fresh juice extract of *Croton macrostachyus* leaves were respectively prepared by dissolving the extracts in liquid paraffin. Freshly prepared ointment was used for the experiment (Omale and Isaac, 2010).

3.7.2 Wound induction and dressing

32 male Wister rats were randomly grouped in to four groups each containing eight rats. The rats were anaesthetized with diethyl ether socked cotton inside the desiccator. The skin on the back were shaved and sterilized with 70% ethanol. Full thickness excision wound of approximately 300mm^2 were prepared by excising the dorsal skin of all rats and then, immediately treated with their respective group. Group I was serve as control (excision wound of rats of control group was treated with liquid paraffin), Group II was treated with Surgicel gauzing (standard), Group III was serve as test group that was treated with fresh juice preparation and Group IV was served as test group that was treated with ethanol extract.

3.7.3 Morphological study

The wounding day was considered as day 0. The measurement of wound area was done on 1st, 4th, 9th and 14th day following the initial wounding using transparent paper and permanent marker. The recorded wound area was measured with graph paper. Photographic picture was taken in every three days for 20 post wounding days. The period of the epithelization was calculated as the number of days required for falling of the dead tissue remnants without any residue of raw wound (Anitha et al., 2009).

3.7.4 Hydroxyproline Estimation

Principle

The determination of collagen in biological tissues usually requires acid hydrolysis and measurement of the hydroxyproline released. The oxidation of hydroxyproline by Chloramine-T gives a stable product pyrrole, which reacts with Para dimethyl amino benzaldehyde to form a stable chromophore.

This technique is based upon three main steps: hydrolysis, oxidation, and development of a chromophore which gives color to certain organic compounds .Hydrolysis is usually carried out at 120^{o}C and the oxidizing agent is chloramine-T (N-chloro-4-toluenesulfonamide, sodium salt). In the oxidation process, the pyrrolidine ring of hydroxyproline undergoes oxidative dehydrogenation to a pyrrole ring. This effect usually occurs within 20 minutes at room temperature. Addition of Ehrlich's reagent produces a quinoid compound that is colored. The development of the chromophore is obtained within 20-25 minutes of incubation at 60-65^{o}C. The absorbance is read at 550-570 nm within three hours of chromophore development. After three hours the chromophore starts to break down. This results in decreased absorbance values (Ignat'eva et al., 2007).

Procedure

In the 10th post wounding day three rats from each group were sacrificed and the granulation tissue was taken. The granulation tissue was preserved in deep freeze till further experiment. The granulation tissue preserved was dried at 60^{o}c in oven for 24 hours. Then, it was weighed and kept in glass stoppered test tubes. After addition of 6N HCL in each test tube so that it contains 40mg of the dried granulation tissue per milliliter of acid, they were incubated at 130^{o}c for 12 hours in oven for hydrolysis. The samples were removed from the oven and kept close until they were cooled at room temperature. 10N NaOH was added drop by drop to neutralize the acid in the presence of 1% phenolphthalein until the color changed faint pink color. The hydrolysate was mixed well by inverting the closed tubes and 100 µL of each hydrolyzed sample was taken in to

labeled glass test tubes, which represents 4mg of the original dried granulation tissue. 4.50 mL of double distil water was added to each test tubes to a total volume of 4.60mL. In a test tube marked "0" (blank) 4.60mL of double distil water was added. Hydroxyproline standard solutions containing 1, 1.5,3, and 6 µg per mL double distil water were prepared from sock solution containing 1mg of hydroxyproline powder in milliliter of double distil water. 100 µL of these solution were kept in labeled test tubes and 4.50mLof double distil water was added to each standard solutions to a total volume of 4.60mL. Then to each test tube (all above) 2.0 ml of 0.2 M freshly prepared chloramine T was added and test tubes are screwed and kept at room temperature for 25 minutes. 3.0 ml of perchloric acid was added to stop oxidation by chloramine T. Finally 0.6 ml of 5% Para dimethyl amino benzaldehyde dissolved in n-propanol was added to each test tube; mixed well and incubated at 60^{o}C for 30 minutes. The absorbance was read at 562 nm using ELISA plate wells and the amount of hydroxyproline were calculated from the standard curve.

3.7.5 Histological Examination

Principle

When tissues are disrupted following injury, collagen is needed to repair the defect in order to restore anatomic structure and function. It is very important in all stages of the wound healing process as it provides strength and integrity to all tissues (Mayer & Willemsteijin, 2008). It would be advantageous if the collagen fibers could be evaluated or measured in order to deeply understand how the collagen is synthesized and re-organized in the wound healing process. The most used method by researchers all around the world in histopathological study of wound healing process is by using the standard Haematoxylin and Eosin (H&E) staining.

Procedure

In the 10^{th} post wounding day wound area from two rats per group was excised. The excised wounds that kept in 10% buffered formalin were used for histological examination. After several steps of alcohol dehydration 4µm thick sections of paraffin embedded tissues were prepared using microtome and sections were stained with haemotoxyline and eosin. The stained sections of the skin were carefully examined under binocular compound microscope (Axiostar 1122-100

USA). Photographic pictures were taken at magnification 40X objectives using ZEISS MC 80 DX (ZEISS, Germany) automated photo camera loaded with Kodak professional ProFotoXL 100 film. The slides were evaluated by pathologist under microscope for collagen deposition, cell differentiation and vascularization.

3.8. Statistical analysis

Statistical analysis was performed using Statistical Package for the Social sciences (SPSS) 15.0 packages (SPSS, USA) and Excel data sheet. Values were expressed as mean ± SEM and mean differences. Descriptive and multiple comparisons were used to compare values. A p value less than 0.05 was considered statistically significant.

4. Results

4.1. Extracts

The extraction of shade dried *Croton macrostachyus* leaves using 70% ethanol solution was carried out for two rounds and the percentage yield of ethanol free dried extract obtained was presented below (table 4.1).

$$\text{Percentage yield} = \frac{\text{Wt. of dried \& collected extract}}{\text{Wt. of the sample (powder)}} \times 100$$

Table 4.1 Percentage yield of ethanolic Croton macrostachyus leaves extract.

Sample weight(g)	Extract weight (g)	Yield (%)
350	**51**	**14.57**

The preparation of juice extract of *Croton macrostachyus* was done using 400g washed and mortar crushed fresh leaves socked in 1.5 liters of distil water. 106.50mL of concentrated juice was obtained.

4.2. In Vitro Experimentation

4.2.1 The effect of Croton macrostachyus leaves extracts on platelets aggregation

The effect of *Croton macrostachyus* leaves extracts on platelets aggregation was measured spectrophotometrically by absorbance values .Decrease in absorbance value was used to quantify the aggregation of platelets. There was decrease in absorbance for increasing doses of ethanolic leaves extract of *Croton macrostachyus*. The mean difference of absorbance for different doses (0.125, 0.25, 0.5, 5.0 mg/mL) of ethanol extract was 0.026, 0.067, 0.135, 0.180 and 0.204 respectively. This values were significant ($p < 0.05$) compared with negative control where as mean difference of absorbance of that of the aqueous juice extract was not significant ($p<0.05$) compared with negative control. All the doses of ethanolic extract showed significant decrease in absorbance as compared to aqueous juice extract ($p<0.05$). The ethanolic extract showed significant decrease in absorbance (increased platelets aggregation) as the dose of extract increase (table 4.2).

Table 4.2 Platelet aggregation induced by Croton macrostachyus leaves extracts compared with negative control.

Groups	*Dose*	*Absorbance in Mean ± Standard error*	*Mean difference*
Negative control	-	0.800 ± 0.008	
Ethanol extract	0.125 mg/mL	0.774 ± 0.012	0.026*
	0.25 mg/mL	0.733 ± 0.009	0.067*
	0.5 mg/mL	0.665 ± 0.010	0.135*
	1 mg/mL	0.620 ± 0.007	0.180*
	5 mg/mL	0.596 ± 0.005	0.204*
Aqueous Juice extract	-	0.801 ± 0.003	-0.001

*p< 0.05 as compared to negative control and aqueous juice extract

Mean difference= mean absorbance of negative control- mean absorbance of sample

4.2.2. Effect of ethanolic extract of *Croton macrostachyus* leaves on time dependent platelets aggregation

In the former result it was shown that ethanolic extract had aggregating effect. The time dependent effect on platelets aggregation was further evaluated using 5mg/mL dose. 5mg/mL of ethanolic extract of *Croton macrostachyus* showed a significant decrease in absorbance of PRP as the time of contact increase. The mean absorbance was decreased from 0.60 to 0.43 respectively after 1 minute and 12 minutes for five replicates. The decrease in absorbance quantifies increase in platelets aggregation and this reflects occurrence of platelets release reactions which lead to self activation of platelets (figure 4.1).

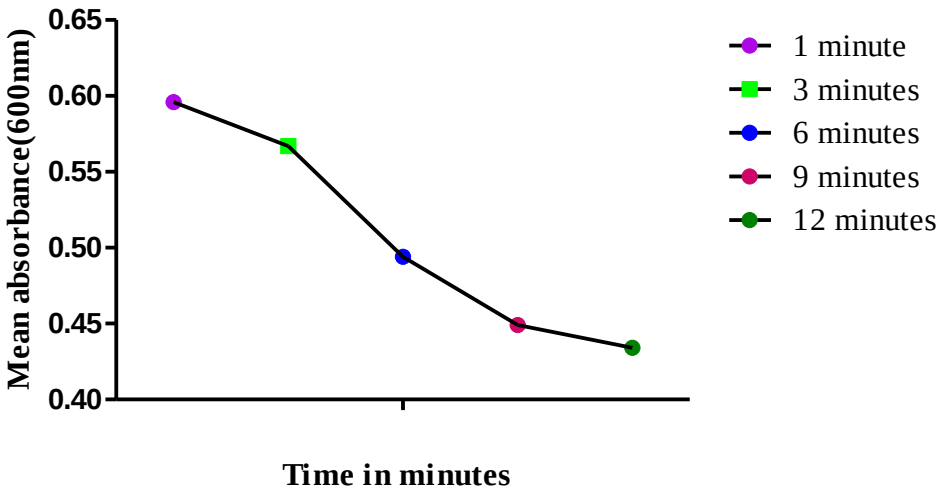

Figure4.1 The time dependent decrease in absorbance of PRP treated with 5mg/mL of ethanolic extract of Croton macrostachyus leaves.

4.3. In Vivo Experimentations

4.3.1. The effect of *Croton macrostachyus* leaves extracts on wound contraction and period of re-epithelization.

Treatment with both ethanolic extract and aqueous extract of the leaves of *Croton macrostachyus* leads to a significant increase ($p < 0.05$) in the rate of wound contraction compared with negative control, which was described by mean of wound percentage contraction ± SEM. The rate of wound contraction of ethanolic extract treated group compared with standard group was significantly higher ($p<0.05$). It was also shown that group treated with ethanolic extract of *Croton macrostachyus* had higher percentage of wound contraction, compared to aqueous extract of *Croton macrostachyus* (table 4.3).

$$\text{Percentage of wound closure} = \frac{\text{wound area on day 0} - \text{wound area on day n}}{\text{Wound area on day 0}} \times 100$$

Where, n =number of days 1^{th}, 4^{th}, 9^{th}, and 14^{th} day.

Table.4.3. Effect of topical application of ointments containing ethanolic and fresh juice extracts of Croton Macrostachyus leaves on wound contraction of excision wound.

Group	Mean percentage of wound contraction ±SEM				Period of epithelization (days)
	1st day	4th day	9th day	14th day	
Control	11.93 ± 2.20	34.73 ± 1.06	55.67 ± 1.00	82.80 ± 0.31	24
Standard	***	47.60 ± 0.51*	76.40 ± 0.50*	90.80 ± 0.22*	21
Ethanol ext.	31.07 ± 0.65*	51.27 ± 0.88**	82.07 ± 0.56**	95.27 ± 0.36**	17
Aqueous ext.	27.27 ± 0.50*	44.33 ± 1.43*	76.13 ± 0.50*	89.13 ± 0.71*	20

*p< 0.05 as compared to negative control, **p<0.05 as compared to standard group, *** surgicel gauzed and measurement was not done.

4.3.2. Hydroxyproline Estimation

4.3. 2.1 Hydroxyproline standards

The mean absorbance of hydroxyproline standards at 562nm was obtained by subtracting mean absorbance of blank from the mean OD readings of the standards. It was summarized in table 4.1 as shown below.

Table. 4.4. Mean absorbance of hydroxyproline standards.

Hydroxyproline standards (µg)	0	1.0	1.5	3.0	6.0
Mean absorbance	0	0.069	0.096	0.197	0.421

Mean absorbance of blank was taken as zero and mean absorbance of blank was subtracted from mean absorbances of standards

Linear regression analysis of the hydroxyproline standards and their respective absorbance to identify the relationship between hydroxyproline (µg) and absorbance at 562nm was obtained as shown below in figure 4.2.

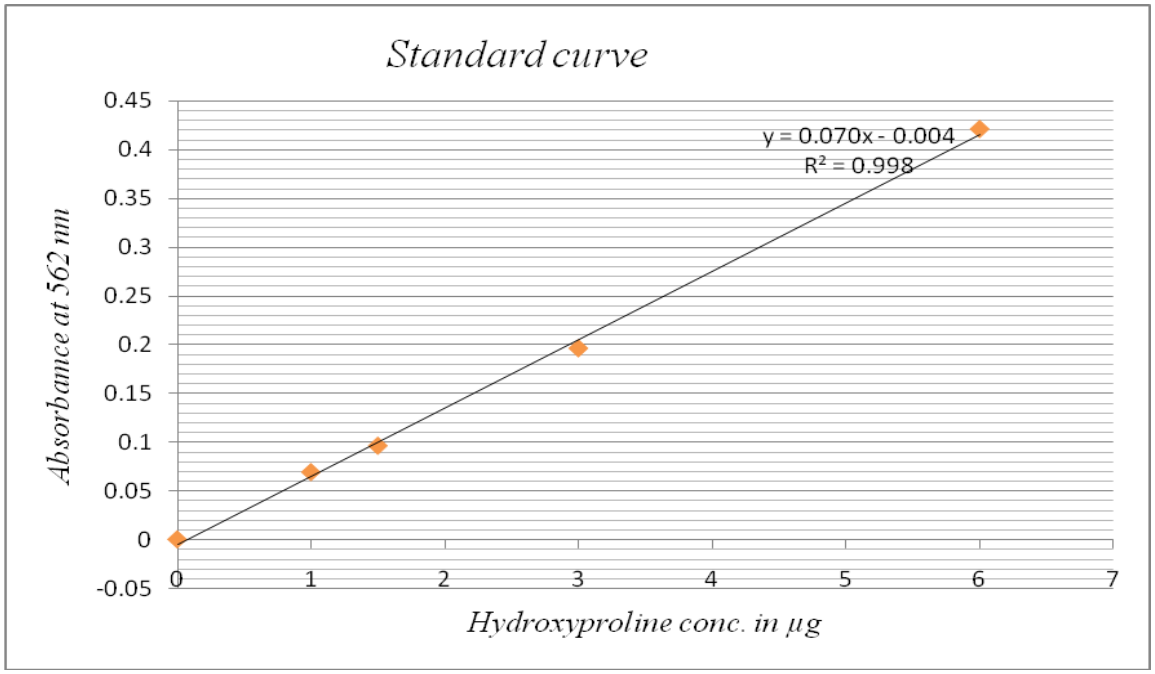

Figure 4.2 Hydroxyproline standard curve and the least squares determination of the standard curve equation relating hydroxyproline (µg) to absorbance. Inserts show the results of regression analyses.

By using the regression curve of the hydroxyproline standards above, the amount of hydroxyproline in each test samples can be determined. The appropriate relationship is:

Sample hydroxyproline= (sample absorbance+0.004)/0.070

Since the amount of sample in the final colorimetric reaction represents a proportion of 0.4 mg/ml of the initial dry granulation tissue, hence, the amount of hydroxyproline (µg) per dry granulation tissue was computed by dividing the amount of hydroxyproline in the samples to 0.4 mg of dry granulation tissue.

4.3.2.2 Hydroxyproline Content of the samples

The hydroxyproline content of the granulation tissue excised from the wound area in the 10th post wounding day was determined spectrophotometrically. The hydroxyproline content in µg was quantified per mg of dried granulation tissue. The animals treated with ointment containing 10% (w/w) ethanolic extract indicated significantly high ($P < 0.05$) levels of hydroxyproline (25.118µg/mg) as compared to negative control (16.083 µg/mg) and the standard (22.404µg/mg). The animals treated with aqueous juice extract and standard also contain significant level ($p<0.05$) of hydroxyproline as compared with negative control (table 4.5). An increase in hydroxyproline content indicates increased collagen synthesis, thus, enhanced wound healing.

Table4.5. Effect of topical application of ethanolic and fresh juice extracts of Croton macrostachyus leaves on hydroxyproline content of the excised granulation tissue.

Groups (N=3)	Hydroxyproline (µg/mg)
control	16.083 ± 0.195
standard	22.404 ± 1.690*
Ethanol extract	25.118 ± 0.490**
Aqueous juice extract	21.583 ± 0.399*

*p<0.05 Compared with negative control

**p<0.05 compared with negative control and the standard

4.3.3. Histological Examination

Histological wound tissue observation and description in haemotoxyline and eosin stained 4µm thin sections was done by pathologist for both treated and control groups under the microscope. Wounds taken from rats treated with ethanolic extract, aqueous Juice extract and standard demonstrated greater degree of collagen deposition and extracellular matrix development (black arrow in figure 4.3) compared to negative control. Ethanolic extract treated group showed enhanced neovascularization and epithelization (red and white arrow in figure 4.3).

Wound taken from negative control demonstrated more population of fibroblasts indicating that the wound tissue was at early stages of collagen synthesis and matrix development (blue arrow at figure 4.3).

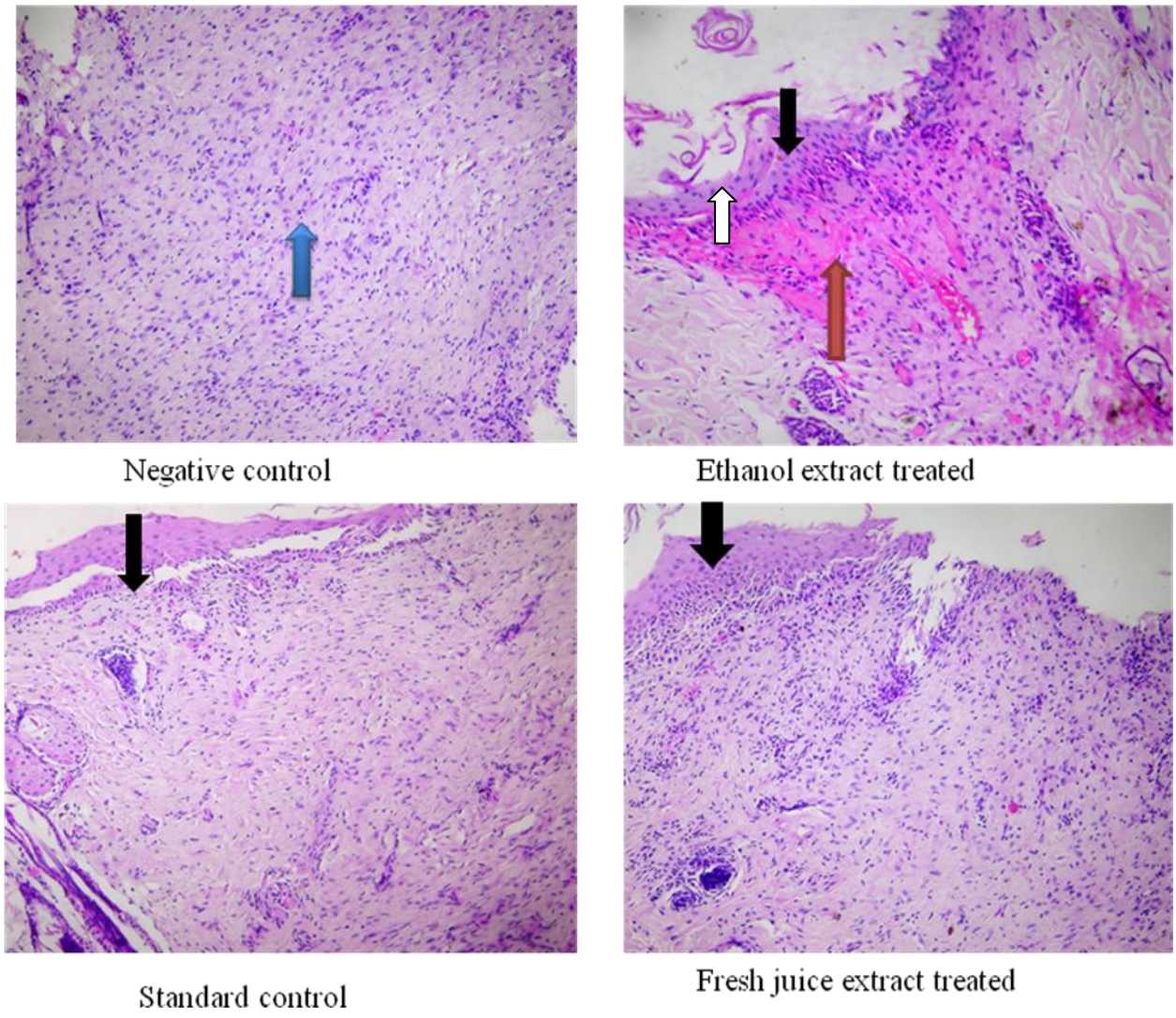

Fig. 4.3 Histopathological examination result photographs of slides of wound tissue

5. Discussion

Platelet activation in response to tissue damage and vascular exposure results in the formation of a platelet plug and blood clot to provide hemostasis and the secretion of biologically active proteins. These proteins, in turn, set the stage for tissue healing, which includes cellular chemotaxis, proliferation, and differentiation; removal of tissue debris; angiogenesis; and the laying down of extracellular matrix and regeneration of the appropriate type of tissue (Marx 2004). The physiological agonists of platelets include collagen from exposed endothelium, epinephrine from nerve endings and thrombin from the coagulation cascades. Furthermore ADP, serotonin and thromoxane A_2 are physiological platelets agonists from the degranulation of platelets granules (Jennings 2009). A number of research works revealed that several agonists can initiate in vitro platelet aggregation. Examples of such agonists are ADP, arachidonic acid, collagen and epinephrine.

In Vitro the ethanolic extract of Croton macrostachyus induce significant platelets aggregations as compared to negative control where as the aqueous juice extract does not show significant aggregations. This finding agrees with a study on *Croton ruizianus*, plant under the same genera by Piacente et al. (1998) that described methanolic extract of aerial part of the plant was found to promote platelet aggregation. This suggests that the ethanolic extract has agonist effect on platelet aggregations and may have a similar effect to commercial haemostatic drugs such as Syvex patch, Chitosan based and Quick clot that enhance the platelet aggregation phase of hemostasis (Seyednejad *et al.,* 2008). Therefore, this study provides scientific basis for the use of fresh juice from the leaves of *croton macrostachyus* as a haemostatic agent in traditional medicine.

There are two types of platelets agonists, strong agonists such Collagen, thrombin, thromboxane A2 and phorbol myristate acetate that directly induce platelet aggregation, thromboxane A2 synthesis and platelet granule secretion. Weak agonists include ADP & epinephrine that induce platelet aggregation without inducing secretion (Patel et al., 2003). Strong agonists induce the release of ADP and thromboxane A2 which interact with their specific receptors on platelets, amplify the aggregation response of platelets (Manash, 2011; Patel *et al.,* 2003).

Strong agonists such as collagen, thrombin and thromboxane A2 are physiological agonists where as phorbol myristate acetate not. Estensen and White, (1974) described that Phorbol myristate acetate (PMA) is the active ingredient of croton oil isolated from *Croton tiglium*. It is a potent stimulus of platelet aggregation and able to induce platelet aggregation in the presence of agents which inhibited aggregation and is capable of triggering the platelet release reaction.

In the present study, the ethanolic extract induced platelet aggregation that significantly increased as contact time with platelets rich plasma (PRP) increased and this effect had similarity to strong platelets agonists that induce platelets aggregation by enhancing platelets release reactions. Furthermore the extract may have phorbol myristate acetate, phytochemical constituent in *Croton tiglium* (plant under the same genera to *Croton macrostachyus*).

Wound healing is the process by which damaged tissue is restored as closely as possible to its normal state and wound contraction is the process of shrinkage of area of the wound. Any reagent which accelerates the process is promoter of wound healing (Divya *et al.,* 2011). In the present study, *Croton macrostachyus* showed positive effect on wound healing, with significant increase in wound contraction. The efficacy of this plant on wound healing may be explained by its haemostatic effects which had been verified by its agonist effect on platelets aggregation. To this end, the plant was traditionally claimed for its antimicrobial role (Mairura, 2007), hence, its efficacy on wound healing in the present work may be partly due to its antimicrobial role. Getachew *et al.,* (2011) verified that the essential oil from *Croton macrostachyus* have antibacterial activity in selected pathogenic bacteria. Although it was not studied in detail the chemical constituent of *Croton macrostachyus* include phenolic compounds such as flavonoids (Amare, 2010). Recent studies shown that phytochemical constituents like flavonoids and other phenolic compounds have multiple biological effects such as antioxidant property, anti-inflammatory actions and antibacterial actions (Divya *et al.,* 2011). Therefore, the extract of *Croton macrostachyus* leaves may have multiple effects on promoting wound healing. This study result may have similarity with other study done on Ankaferd Blood Stopper (ABS), Turkish herbal medicine extract that is used for controlling bleeding and have multiple therapeutic benefits, because of its anti-infective, antineoplastic, and wound healing properties (Yavuz et al., 2010).

Collagens are the most abundant proteins in the mammalian body and it is well recognized that collagens fulfill an important role in maintaining the structural integrity and healing of wound. (Nikhat and Abdullah, 2003. Many studies attempt to quantify the amount of collagen changing and orientation in any stages of wound healing. Collagen measurement is normally used for application to pharmacological products efficacy test and as toxicity (Ukong *et al.,* 2008).

Tissue hydroxyproline level is the best indicator of collagen synthesis. Hydroxyproline is an amino acid and a sub product of collagen synthesis. Hydroxyproline level shows a parallel increase with collagen synthesis (Mustafa *et al.,* 2003). In the present study the measurement of hydroxyproline concentration, which come from the breakdown of collagen showed a significant increase in both samples treated groups and surgicel treated group compared to a negative control. The increase in hydroxyproline content in the granulation tissue could be indicative to the presence of higher collagen content and its turnover leading to rapid wound healing.

Histopathological studies of wound healing process are normally used for evaluation the efficacy of pharmacological products which promote new collagen fibers formation and wound healing (Ukong *et al.,* 2008). In the present study histopathological examinations revealed better degree of collagen deposition, matrix development and neovascularization in treated group compared with negative control.

6. Conclusion

Croton macrostachyus had various traditionally claimed medicinal values which include hastening blood clotting and promoting wound healing in fresh wound. This study showed that the ethanolic extract of *Croton macrostachyus* leaves had platelets aggregation effect in vitro that verifies its role on haemostasis. Moreover, ethanolic extract of the plant leaves promote wound healing in vivo that could be explained partly by its procoagulant activity. The present of flavonoids and other phenolic compounds may be additional reasons, because these compounds have multiple roles in promoting wound healing. And the aqueous juice extract had promoting effect on wound healing that could be explained by the multiple chemical constituents which are of water soluble.

7. Recommendations

For further research work the following are recommended:

- ✓ *Croton Macrostachyus* has procoagulant properties and wound healing activities but further work needs to be done on safety profile before it can be recommended.
- ✓ The active chemical ingredients of *Croton Macrostachyus* must be extracted and further characterized to prove the different claims of its traditional medicinal values.

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